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GLUCOCORTICOID EFFECTS ON GLUCOSE TRANSPORT AND TRANSPORTER GENE EXPRESSION IN L6 MUSCLE CELLS

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Summary: We questioned whether glucocorticoids alter the known potent effect of serum to increase glucose transport and GLUT-1 expression in cultured L6 myotubes. Myotubes were exposed to serum-free medium for 24h in the presence or absence of 300 nM dexamethasone and subsequently treated for 8h with 10% serum or serum free medium in the continued presence or absence of the glucocorticoid. Dexamethasone did not effect the serum-induced increase in GLUT-1 mRNA. However, dexamethasone increased GLUT-1 mRNA 2-3 fold versus the absence of dexamethasone after serum withdrawal. Dexamethasone also resulted in higher GLUT-1 protein levels and ameliorated the loss of glucose transport activity seen after serum withdrawal.

Several reports provide evidence that the expression of the GLUT-1 facilitative glucose transporter gene is increased under a variety of stress conditions both in cultured cells (1-4) and *in vivo* (5-8). Since increased circulating glucocorticoids represent an integral part of the normal physiologic response to stress, it might be hypothesized that glucocorticoids act, at least in some cell types, to upregulate the expression of this glucose transporter isoform. On the other hand, glucocorticoids are well known to increase glycemia and cause in insulin resistance *in vivo*. In fact, in primary cultured adipocytes, glucocorticoids were shown to decrease glucose transporter number (9,10) and specifically GLUT-1 mRNA and protein (11), possibly through inhibition of transcription (11).

To further study the cellular effects of glucocorticoids, we examined the effects of dexamethasone on glucose transport and transporter gene expression in L6 muscle cells after spontaneous differentiation to myotubes which express both GLUT-1 and GLUT-4. We reasoned that if glucocorticoids impair GLUT-1 transcription, then dexamethasone might inhibit the known potent effect of serum to increase GLUT-1 levels in these cells (12).

MATERIALS AND METHODS

Materials: L6 myoblasts were kindly supplied by Dr. Amira Klip at the University of Toronto. Plasmids prGT-1, which contains a full length insert encoding the rat GLUT-1 protein (13), and pSM1-1-1, which contains a full length insert encoding the rat GLUT-4 protein (14) were gifts

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from Dr. Morris Birnbaum at Harvard Medical School. Rabbit polyclonal antibody directed against carboxy-terminal sequences of rat GLUT-1 (RaGluTrans) was obtained from East-Acres Biologicals, Southbridge, Ma. Dexamethasone sodium phosphate was obtained from Elkins-Sinn, Inc., Cherry Hill, N.J. α-minimal essential medium (α-MEM) was obtained from GibcoBRL, Gaithersburg, Md. Fetal bovine serum was obtained from HyClone, Inc., Logan, Ut. Other reagents were obtained commercially from standard sources.

Cell culture: Cells were grown in α-MEM supplemented with 10 mg/ml penicillin, 10 mg/ml streptomycin, and 25 mg/ml amphotericin B at 37°C under 5% CO_2. Cells were allowed to reach confluency and differentiate in 2% fetal bovine serum following which serum concentrations were changed as indicated.

Glucose transporter mRNA levels: RNA was extracted and GLUT-1 and GLUT-4 mRNA levels were measured as we previously described (15). Cells were homogenized in ice cold guanidine isothiocyanate buffer and mRNA prepared by centrifugation through $CsCl_2$. Specific mRNA levels were determined by Northern Blot analysis using [^{32}P] labeled full length rat GLUT-1, GLUT-4, and GAPDH cDNA probes. Sequential hybridizations to the above probes were carried out after erasing blots by washing in water at 95°C. mRNA levels were quantitated by laser densitometry and the results normalized to total RNA and GAPDH mRNA as a housekeeping marker. Each sample represented RNA from cells grown in one T-75 flask.

Immunoblot analysis: Cells were washed in phosphate buffered saline, scraped in 1 ml cold RIPA buffer (50 mM Tris, pH 7.4., 150 mM NaCl, 1% Triton X-100, 1% Sodium deoxycholate, and 0.1% SDS) and sonicated for 7 seconds. Sonicates were agitated at 4°C for 30 min, and spun at 50,000 x g for 30 min. The supernate containing solubilized membrane protein was frozen at -70°C until used. Protein was determined by the Lowry technique. Western Blot analysis was carried out as we previously described (15). 50 ug samples of solubilized protein extracts were separated on 10% polyacrylamide, transferred to nitrocellulose, and incubated with antibody against GLUT-1 at a dilution of 1:1500 for 8h, washed, and incubated with 0.16 mCi/ml of [^{125}I]protein A for 2h. All incubations were performed at room temperature. The nitrocellulose filters were stained with amido black to ascertain even protein loading. Each lane or each "n" represented protein from one T-75 flask.

Glucose transport activity: D-glucose transport was measured as cytochalasin B inhibitable [^{3}H]2-deoxyglucose uptake. Cytochalasin B specifically inhibits facilitative glucose carriers so the difference between uptake in the presence or absence of this agent reflects facilitated transport (16). Cells were grown and uptake determined in 6 well plates so that each individual measurement represented the difference between the means of triplicate determinations in the presence (3 wells) and absence (3 wells of the same plate) of cytochalasin B. Since separate wells were required (plus or minus cytochalasin B), our uptake experiments included [14C]L-glucose to correct for trapping of D-glucose tracer in individual wells. Transport was measured over 5 minutes (uptake was found to be linear with time for at least 10 minutes) under conditions wherein transport, not phosphorylation is rate limiting for uptake of this monosacharide (17).

Statistics: Data were analyzed by unpaired t-test corrected for the number of comparisons by the Bonferroni method.

RESULTS

L6 myoblasts were grown to confluency in 2% serum. Consistent with prior reports (18), by three days post-confluency myotubes began to form and by 7 days the cell mass was noted to consist almost completely of differentiated myotubes which expressed both GLUT-1 and GLUT-4 mRNA.

We subjected 7 day post-confluent L6 myotubes to 24h withdrawal from 2% serum in the presence or absence of dexamethasone, 300 nM (the concentration reported in adipocyte studies), followed by 8h exposure to 10% serum or serum-free medium in the continued presence or absence of the glucocorticoid. GLUT-1, GLUT-4, and GAPDH mRNA levels were then assessed by Northern Blot analysis (figure 1). As expected, 10% serum markedly increased

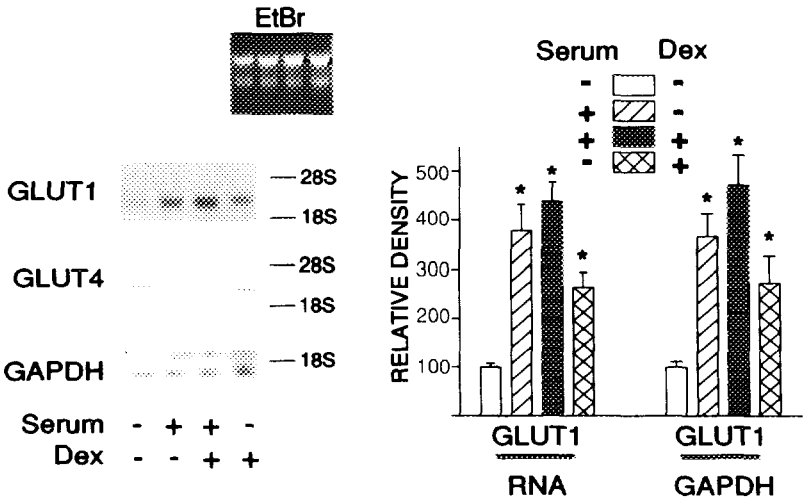

Figure 1. Effect of serum and 300 nM dexamethasone (dex) on glucose transporter mRNA levels measured by Northern Blot analysis. L6 cells were grown in 2% serum until 7 days post-confluency and myotube formation at which time cells were placed in serum-free medium (time 0). Dex was present (+) or absent (-) from time 0 to 32h. Cells were esposed to 10% serum (+) or serum-free medium (-) from time 24h to 32h. Cells were harvested at 32h, total RNA prepared, and specific mRNA levels determined. Equal loading was confirmed by the appearance of the ethidium bromide (EtBr) stained gel. Blots were hybridized to GLUT-1 cDNA, erased and sequentially hybridized to GLUT-4 and GAPDH cDNAs. A representative experiment is shown along with the quantitative results of this and other experiments. Values represent mean ± SEM, n=6, *p < 0.05 versus the absence of dex and serum.

GLUT-1 mRNA levels versus serum-free medium. However, we were surprised that dexamethasone, if anything, enhanced this effect. Further, after serum withdrawal, 32h of dexamethasone alone resulted in increased GLUT-1 mRNA levels compared to the absence of dexamethasone. These effects were the same whether the GLUT-1 data were normalized to total mRNA or to GAPDH used as a "housekeeper" message (figure 1). In contrast to the effects of serum and dexamethasone on GLUT-1 mRNA levels, GLUT-4 levels did not change upon serum exposure and were not affected by dexamethasone. The effect of 10% serum to increase GLUT-1 mRNA post-24h of serum withdrawal was noted in an additional experiment to occur within 1h (335% ± 37, n=3 of pre-serum levels of 100 ± 32, n=4). This suggests that this effect is likely mediated, at least in part, through increased transcription.

In further experiments, we examined the effect of 24h of serum withdrawal in the presence or absence of 300 nM dexamethasone on GLUT-1 protein levels in L6 myotubes. Immunoblot studies (figure 2), revealed that GLUT-1 protein levels were approximately 2-fold greater in the presence versus absence of 300 nM dexamethasone. Quantitative levels of GLUT-1 in arbitrary units normalized to rat brain membrane protein included on each blot were (mean ± SEM, n=4) 198 ± 37 for dexamethasone-treated cells compared to 100 ± 8 for cells unexposed to dexamethasone (n=4, $p < 0.05$).

To determine whether these changes in GLUT-1 protein were associated with altered glucose transport activity, we measured 2-deoxyglucose uptake by L6 myotubes subject to 24h

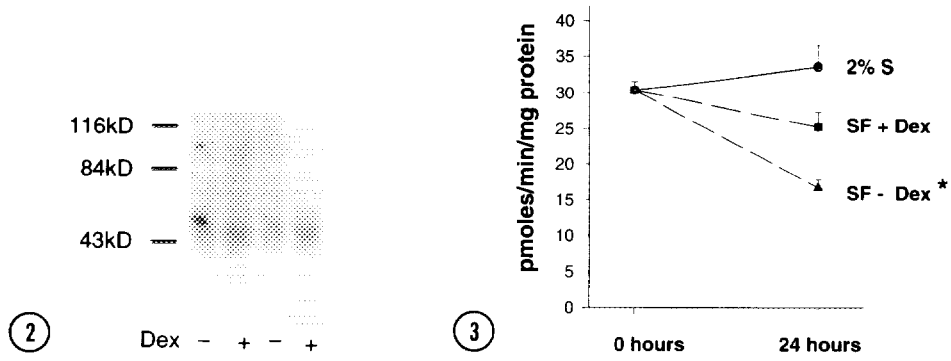

Figure 2. Effect of dexamethasone (dex) on GLUT-1 protein levels in L6 myotubes after serum withdrawal. Cells were grown to 7 days post-confluency in 2% serum and exposed to serum-free medium for 24h in the presence (+) or absence (-) of 300 nM dex. 50 μg solubilized protein extracts were run in each lane. Data are from 2 separate sets of dex-treated or untreated cells and are representative of 2 more sets. Quantitative data are given in the text. Broad bands due to glycosylation are typical for GLUT-1.

Figure 3. Glucose transport activity measured as cytochalasin B inhibitable [^{3}H]2-deoxyglucose uptake in cells grown to 7 days post-confluency in 2% serum before serum withdrawal (time 0) or after 24h of continued maintenance in 2% serum (2% S) or treatment with serum-free medium with (SF + Dex) or without (SF - Dex) 300 nM dexamethasone. Values represent mean ± SEM, n=4, *$p < 0.05$ versus SF + Dex.

serum withdrawal in the presence or absence of dexamethasone (figure 3). Dexamethasone treatment did not completely prevent the loss of 2-deoxyglucose uptake after serum withdrawal, but did increase 2-deoxyglucose uptake by 51% relative to the absence of the glucocorticoid. Although this change was significant, a separate experiment was performed comparing only cells exposed to serum deprivation for 24h in the presence or absence of dexamethasone. 2-deoxyglucose uptake in dexamethasone-treated cells was increased by 52% compared to untreated cells (n= 8 for each group, $p < 0.01$) confirming the results in in figure 3.

DISCUSSION

Dexamethasone treatment (relative to the absence of glucocorticoid) of L6 myotubes subjected to serum withdrawal resulted in higher levels of GLUT-1 mRNA and protein and increased glucose transport activity. Klip et. al. (19) previously reported loss of glucose transport activity in L6 myotubes following serum deprivation and observed that this effect was reduced by cyclohexamide suggesting a dependence on protein synthesis. Our current data indicate that dexamethasone also ameliorates this loss of transport activity and suggest that the mechanism at least in part involves preservation of GLUT-1 expression. Hence, our data taken together with the findings of Klip, et. al. raise the possibility that dexamethasone may act by preventing the production of a protein inhibitor of GLUT-1 biosynthesis.

Our data do not show an inhibitory effect of glucocorticoids on GLUT-1 mRNA and protein levels in L6 muscle cells as has been seen in primary adipocytes (11). This effect in adipocytes was postulated to result from impaired transcription, since adipocyte GLUT-1 mRNA

stability was not affected by dexamethasone as determined by blocking new transcription with actinomysin D. Hypothetically, impaired GLUT-1 transcription might occur through one or more glucocorticoid response elements in the rat GLUT-1 promoter, one of which is located immediately at the transcription initiation site (12). If the rapidity and magnitude of the effect of serum to increase GLUT-1 in L6 muscle cells, is taken as evidence that this effect is mediated through increased transcription, then our data suggest that glucocorticoids do not inhibit GLUT-1 transcription in these cells.

If serum deprivation is viewed as a form of cell stress, then our findings are consistent with a proposed role for GLUT-1 as a stress responsive protein (4) in this case upregulated by glucocorticoid exposure. GLUT-1 upregulation has been reported under a variety of stress conditions both in cultured cells and *in vivo*. These conditions include low O_2 tension (1,2), inhibitors of oxidative phosphorylation (2) and inhibitors of glycosylation (3) in L6 cells as well as disruption of intracellular calcium stores or protein glycosylation (4) in L8 myocytes and NIH 3T3 adipocytes (wherein evidence was presented suggesting that the increase in GLUT-1 may be associated with increased expression of other stress responsive proteins). *In vivo* studies demonstrated that starvation increased GLUT-1 levels in skeletal muscle (5) and liver (6) and hypoxia increased cardiac GLUT-1 levels (7) and brain microvessel GLUT-1 density (8). Both starvation and hypoxia represent conditions wherein an increase in circulating glucocorticoids would be expected.

We could not demonstrate an effect of dexamethasone on GLUT-4 mRNA levels in L6 myotubes. This result is compatible with *in vivo* data of Haber et. al. (20) showing no effect of dexamethasone treatment on rat soleus and extensor digitorum longus muscle GLUT-4 expression. GLUT-4 regulation can not be studied in primary adipocytes since these cells rapidly lose the expression of this transporter isoform once removed from the *in vivo* environment (21).

In summary, our data indicate that dexamethasone does not inhibit the serum-induced increase in GLUT-1 expression in L6 myotubes. Dexamethasone does increase GLUT-1 mRNA and protein expression and ameliorates the loss of glucose transport activity seen in L6 myotubes after serum withdrawal.

ACKNOWLEDGMENTS

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REFERENCES

1. Bashan, N., Burdett, E., Hundal, H.S., and Klip, A. (1992) Am. J. Physiol. 262,C682-C690.
2. Bashan, N., Burdett, E., Guma, A., Sargeant, R., Tumiati, L., Liu, Z., and Klip, A. (1993) Am. J. Physiol. 264,C430-C440.
3. Maher, F. and Harrison, L.C. (1991) Biochimica. et Biophysica. Acta. 1089(1),27-32.
4. Wertheimer, E., Sasson, S., Cerasi, E., and Ben-Neriah, Y. (1991) Cell Biology 88:2525-2529.

5. Charron, M.J. and Kahn, B.B. (1990) J. Biol. Chem. 265,7994-8000.
6. Thorens, B., Flier, J.S., Lodish, H.F., Kahn, B.B. (1990) Diabetes 39,712-719.
7. Harik, S.I., Behmand, R.A., and LaManna, J.C. (1994) J. Appl. Physiol. 77(2),896-901.
8. Sivitz,W.I., Lund, D.D., Yorek, B., Grover-McKay, M., and Schmid, P.G. (1992) Am. J. Physiol. 263,E562-E569.
9. Garvey, W.T., Huecksteadt, T.P., Lima, F.B., and Birnbaum, M.J. (1989) Mol. Endocrinol. 3,1132-1141.
10. Carter-Su, C. and Okamoto, K. (1985) J, Biol, Chem, 260,11091-11098.
11. Garvey, W.T., Huecksteadt, T.P., Monzon, R., and Marshall, S. (1989) Endocrinology 124,2063-2073.
12. Williams, S.A., Birnbaum, M.J. (1988) J. Biol. Chem., 263,19513-19518.
13. Birnbaum, M.J., Haspel, H.C., and Rosen, O.M. (1986) Proc. Natl. Acad. Sci., USA, 83,5784-5788.
14. Birnbaum, M.J. (1989) Cell 57,305-315.
15. Sivitz, W.I., DeSautel, S.L., Lee, E.C., and Pessin, J.E. (1992) Metabolism 41,1267-1272.
16. Simpson, I.A. Cushman, S.W. (1986) Annual Review of Biochemistry 55,1059-1089.
17. Klip, A., Logan, W.J., and Li, G. (1982) Biochim. Biophys. Acta. 687,265-280.
18. Klip, A. and Marette, A. (1992) J. Cellular Biochemistry 48,51-60.
19. Klip, A., Li, G., and Logan, W.J. (1984) Am. J. Physiol. 247,E291-E296.
20. Haber, R.S. and Weinstein, S.P. (1992) Diabetes 41,728-735.
21. Gerritis, P.M., Olson, A., and Pessin, J.E. (1993) J. Biol. Chem. 268,640-644.